TREATMENT FOR INSULIN DEPENDENT DIABETES

FIELD OF THE INVENTION

The present invention relates to a treatment for insulin dependent (type-I) diabetes. More particularly, this invention relates to the use of antibodies recognizing the integrin VLA4 (very late antigen 4) in the prevention of diabetes.

BACKGROUND OF THE INVENTION

Insulin dependent diabetes (also termed type-I diabetes and formerly juvenile onset diabetes mellitus) has been classified during the past two decades as a chronic autoimmune disease. In this disorder, cells producing insulin (β cells) within the pancreatic islets are selectively targeted and destroyed by a cellular infiltrate of the pancreas. This inflammatory infiltrate affecting the islets has been termed insulitis. Cells producing insulin comprise the majority of islet cells but less than 2% of the total pancreatic mass (Castano and Eisenbarth, 1990, [1]; Fujita et al., 1982 [2]; Foulis et al., 1986 [3]). The development of type I diabetes can conceptually be divided into six stages, beginning with genetic susceptibility and ending with complete β cell destruction (Eisenbarth, 1986 [4]). Stage I is genetic susceptibility, which is a necessary but insufficient condition for development of the disease. A hypothetical triggering event (Stage II) leads to active autoimmunity against β cells (Stage III). In Stage III, the β cell mass is hypothesized to decline and immunologic abnormalities such as autoantibodies directed against insulin and islet cytoplasmic antigens are found. Stimulated insulin secretion is still preserved at this

stage. Over a period of years, however, the progressive loss of β cells leads to diminished insulin secretion with intravenous glucose tolerance tests (IVGTT) while the individual is still normoglycemic (Stage IV). Overt diabetes (i.e., diabetes onset or clinical manifestation of disease characterized by hyperglycemia) is Stage V, and can develop years later when approximately 90% of pancreatic β cells are destroyed. In Stage V when overt diabetes is first recognized, some residual insulin production remains (as demonstrated by the presence of the connecting peptide of proinsulin, C peptide, in the serum) but the individual usually requires exogenous insulin for life. Finally, in Stage VI, even the remaining β cells are destroyed and C peptide can no longer be detected in the circulation.

While the initiating factor(s) and specific sequence of events leading to diabetes, including the relative importance of different cell types and cytokines, are still widely debated, a key role is generally recognized for self-antigen reactive T cells (Miller et al., 1988 [5]; Harada and Makino, 1986 [6]; Koike et al., 1987 [7]; Makino et al., 1986 [8]). In addition to T lymphocytes, insulitis is characterized by macrophages, dendritic cells (Voorbij et al., 1989 [9]) and B cells, which may serve as professional antigen presenting cells (APC). Macrophages may also destroy islet β cells themselves by release of cytokines or free radicals (Nomikos et al., 1986 [10]). Thus, autoimmune diabetes relies upon both cellular migration and immune stimulation of newly resident cells.

Cell trafficking to inflammatory sites is regulated by accessory molecules LFA-1, MAC-1 and VLA4 (Larson and Springer, 1990 [11]; Hemler et al., 1990 [12]) on the surface of lymphocytes (LFA-1, VLA4) and macrophages (Mac-

1, VLA4), and by their counter-ligands ICAM (for LFA-1 and MAC-1), and VCAM (for VLA4) which are unregulated by cytokines on vascular endothelium (Larson and Springer, 1990 [11]; Lobb, 1992 [13]; Osborn, 1990,[14]). In addition, VLA4 binds to an extracellular matrix component, the CS-1 domain of fibronectin (FN) (Wayner et al., 1989 [15]). The relative importance of these pathways, for example, LFA-1 and VLA4 on lymphocytes or MAC-1 and VLA4 on monocytes, in controlling cell migration is still a subject of investigation. *In vitro* data suggest that the differential use of these pathways appears to depend upon the activation status of both the leukocytes and endothelial cells (Shimizu et al., 1991 [16]). Their ability to control cell migration to inflammatory sites *in vivo* has been directly demonstrated with monoclonal antibodies (mAbs) to ICAM, MAC-1 or VLA4 inhibiting various animal models of disease (Barton et al., 1989 [17], phorbol ester-induced rabbit lung inflammation; Issekutz and Issekutz, 1991 [18], delayed type hypersensitivity; Issekutz, 1991 [19], adjuvant-induced arthritis; Yednock et al., 1992 [20], transfer of experimental allergic encephalomyelitis (EAE); Lobb, 1992 [21], asthma).

ICAM and VCAM are also found on the surface of macrophages and dendritic cells in lymphoid tissues (Dustin et al., 1986 [22]; Rice et al., 1990 [23]; Rice et al., 1991 [24]). Their distribution on these professional APC is consistent with functional data indicating a role for LFA-1 and VLA4 in T cell activation (Shimuzu et al., 1990 [25], Burkly et al., 1991 [26]). However, numerous other receptor-ligand pairs including CD4/ MHC class II and CD8/MHC class I (Rudd et al., 1989 [27]), CD2/LFA-3 (Moingeon et al., 1989 [287]), CD28/B7 (Harding et al.,

1992 [29]) may also support adhesion or costimulate T cells during T/APC or T/target cell interactions. The specific contributions of these numerous pathways in the development of diabetes is unresolved. Because there are multiple molecular pathways for cell adhesion and T cell activation, it is not possible to predict whether intervention in one or more of these pathways might affect onset or severity of diabetes disease, and, in particular, which of these pathways are crucial or relevant to the disease process.

Antibodies directed to T cells have been utilized in murine and rat models for spontaneous diabetes and adoptive transfer of diabetes to deplete T cells and thus prevent disease (see, e.g., Harada and Makino, 1986 [6], anti-Thy 1.2; Koike et al., 1987 [7], Miller et al., 1988 [5] and Shizuru et al., 1988 [30], anti-CD4; Barlow and Like, 1992 [31], anti-CD2; Like et al., 1986 [32], anti-CD5 and anti-CD8). In addition, an antibody directed to the complement receptor type 3 (CR3) molecule or MAC-1 on macrophages has been utilized to prevent macrophage and T cell infiltration of pancreatic tissue in a murine adoptive transfer model of disease (Hutchings et al., 1990 [33]). It is unknown whether VLA4 is relevant to insulitis or to the activity of islet-specific cells after localization in the pancreas.

Current treatment protocols suggested for type I diabetes have included certain immunomodulatory drugs summarized by Federlin and Becker [34] and references cited therein. A long prediabetic period with immunologic abnormalities and progressive β cell destruction suggests it may be possible to halt β cell loss with immune intervention (Castano and Eisenbarth, 1990 [1]).

Suggested agents/protocols have included certain immunomodulatory and immunosuppressive agents: levamisol, theophyllin, thymic hormones, ciamexone, anti-thymocyte globulin, interferon, nicotinamide, gamma globulin infusion, plasmapheresis or white cell transfusion. Agents such as cyclosporin A and azathioprine which impair T cell activation and T cell development, respectively, have been used in clinical trials (Zielasek et al., 1989 [35]). The most promising results have been achieved with cyclosporin A (Castano and Eisenbarth, 1990 [1]). Federlin and Becker, 1990 [34] suggest, however, that cyclosporin A may not be recommended for general or long-term use because of toxic side effects, at least when given in higher doses. Higher doses of cyclosporin, or in combination with other immunosuppressive drugs, or both, have been associated with the development of lymphoma and irreversible kidney damage (Eisenbarth, 1986 [4]; Eisenbarth, 1987 [36]) Additional studies on other suggested agents are necessary to assess safety and efficacy. Even the cyclosporin A studies show that its efficacy in maintaining remission of diabetes is for one year in about 30-60% of new onset diabetes. Within 3 years, however, remissions are almost invariably lost (Castano and Eisenbarth, 1990 [1]). Treatment protocols after onset of disease are particularly problematic, since, for example, at the time diabetes is diagnosed in humans, insulitis has typically progressed already to a loss of more than 80% of the β cells. Thus, it is possible that cyclosporin A may be preventing further β cell destruction, but so few β cells may be present at the onset of the diabetes that they cannot maintain a non-diabetic state over time (Castano and Eisenbarth, 1990 [1]). Suppression of insulitis and/or prevention of

disease may be more successful if the treatment could start at an earlier phase, i.e., before disease onset.

There are two major prerequisites in order to develop any preventative treatment for diabetes disease: (1) the ability to accurately identify the prediabetic individual and (2) the development of safe, specific and effective preventive treatments. Significant progress has been made in identifying prediabetic individuals, however, much work remains in the development of safe, specific and effective preventive treatments as discussed and reviewed by Eisenbarth and colleagues (see, e.g., Ziegler and Eisenbarth, 1990 [37]; Ziegler et al., 1990 [38]; Ziegler et al., 1990 [39]). It has been possible to identify certain risk factors and at-risk groups for type I diabetes and thus to predict individuals most likely to go on to clinical disease and to estimate the approximate rate of disease onset in these individuals. The ability to identify individuals with susceptibility to diabetes or to predict type I diabetes in the pre-clinical stage by the combination of genetic (HLA typing), immunological (islet and insulin autoantibodies) and metabolic (first phase insulin secretion to intravenous glucose preceding the development of hyperglycemia) markers makes the identification and use of prophylactic immunotherapeutic drugs and protocols possible during the evolution of the autoimmune disease process when β cell destruction is only partial. To date, there has been little success, however, in treating human diabetes. Generally, because human treatment has been used only after onset of the disease, treatment was followed by a temporary complete or partial remission only in a certain number of patients. Since immunosuppressive mechanisms may prevent insulitis and/or diabetes, there is a need for immunosuppressive components

for use in the prediabetic stage. In particular, there is a need for safer and more specifically acting compounds, e.g., monoclonal antibodies, which inhibit entry of effector cells into the pancreas or function of those cell which may have already entered the islets of Langerhans.

It has now been surprisingly discovered that administering an anti-VLA4 antibody significantly reduced the incidence of diabetes, in a rodent model of diabetes disease. The NOD mouse model of diabetes is a well established model directly comparable to human type-I diabetes. Using an adoptively transferred disease experimental protocol, irradiated non-diabetic NOD mice were administered splenocytes from spontaneously diabetic NOD mice for the acute transfer of the disease. These splenocytes were treated with anti-VLA4 antibody before administration and the recipients were also treated for various periods of time after the transfer with anti-VLA4 antibody.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides novel methods for the treatment of insulin dependent (type-I) diabetes in a prediabetic. In particular, the present invention provides a method for the prevention of insulin dependent diabetes comprising the step of administering to a prediabetic individual an anti-VLA4 antibody, such as antibody HP1/2 or a humanized anti-VLA4 antibody derived from HP1/2. Also contemplated is the use of analogous antibodies, antibody fragments, soluble proteins and small molecules that mimic the action of anti-VLA4 antibodies in the treatment of diabetes. In addition, the present invention provides a method for the treatment of diabetes by administering to a mammal, including a human, with a susceptibility to diabetes an antibody capable of binding to the α4 subunit of VLA4 in an amount effective to provide inhibition of the onset of diabetes. Also contemplated is the use of recombinant and chimeric antibodies, fragments of such antibodies, polypeptides or small molecules capable of binding α4/VLA4. Also contemplated are soluble forms of the natural binding proteins for VLA 4, including soluble VCAM-1 or VCAM-1 peptides as well as fibronectin, fibronectin having an alternatively spliced non-type III connecting segment and fibronectin peptides containing the amino acid sequence EILDV or a similar conservatively substituted amino acid sequence. These agents will act by competing with the cell-surface binding protein for VLA4.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the effect of anti-VLA4 antibody (R1-2) and controls on prevention of diabetes after adoptive transfer of spleen cells; the frequency of recipients which became diabetic and day of disease onset are shown for transfer of $2x10^7$ splenocytes from diabetic (D) NOD donors without treatment (closed circles), with a non-specific rat IgG2b treatment (closed triangles), and with R1-2 anti-VLA4 treatment (closed diamonds), as well as for transfer of splenocytes from nondiabetic (Y) NOD donors (open squares); the splenocytes were transferred with R1-2 or rat IgG2b or without mAb, and then R1-2 or rat IgG2b was injected every other day through day 12 post transfer (n=8-10 for all groups).

Figure 2 is a graph depicting the effect of anti-VLA4 antibody (R1-2) and controls on prevention of diabetes after adoptive transfer of spleen cells; the frequency of recipients which became diabetic and day of disease onset are shown for transfer of $3x10^7$ splenocytes from diabetic (D) NOD donors without treatment (closed circles), with a non-specific rat IgG2b treatment (closed triangles), and with R1-2 anti-VLA4 treatment (closed diamonds), as well as for transfer of splenocytes from nondiabetic (Y) NOD donors (open squares); the splenocytes were transferred with R1-2 or rat IgG2b or without mAb, and then R1-2 or rat IgG2b was injected every 3.5 days through day 25 post transfer (n=4-5 for all groups).

Figure 3 is a graph depicting the effect of anti-VLA4 antibody (R1-2) and controls on prevention of diabetes after adoptive transfer of spleen cells; the frequency of recipients which became diabetic and day of disease onset are shown for transfer of $2-3x10^7$ splenocytes from

diabetic (D) NOD donors without treatment (closed circles), with a non-specific rat IgG2b treatment (closed triangles), and with R1-2 anti-VLA4 treatment (closed diamonds), as well as for transfer of splenocytes from nondiabetic (Y) NOD donors (open squares) or for PBS alone (open circles); the splenocytes were transferred with R1-2 or rat IgG2b or without mAb, and then R1-2 or rat IgG2b was injected every 3.5 days through day 25 post transfer (n=5 for all groups).

Figure 4 is a bar graph depicting the effect of anti-VLA4 antibody (R1-2) and controls on the degree of insulitis after adoptive transfer of spleen cells; the frequency of uninfiltrated islets (Grade 0-I infiltrate, stipled bar) and infiltrated islets (Grade II-IV insulitis, solid bar) were quantitated and shown after transfer of cells treated with R1-2, rat IgG2b or without mAb, and then R1-2 or rat IgG2b injected every 3.5 days through day 25 with mice sacrificed when diabetic or on day 26 post-transfer. Pancreatic sections from n=4-5 mice were scored for each experimental group, i.e., Y→Y (non-diabetic donor cells) or D→Y (diabetic donor cells) into non-diabetic (Y) recipients with no mAb treatment, treatment with rat IgG2b or treatment with R1-2.

Figure 5 is a bar graph depicting the effect of anti-VLA4 antibody (R1-2) and controls on the degree of insulitis after adoptive transfer of spleen cells; the frequency of uninfiltrated islets (Grade 0-I infiltrate, stipled bar) and infiltrated islets (Grade II-IV insulitis, solid bar) were quantitated and shown after transfer of cells treated with R1-2, rat IgG2b or without mAb, and then R1-2 or rat IgG2b injected every other day through day 12 post-transfer, then maintained without further mAb injection until sacrificed when diabetic or on

day 29 post-transfer. Pancreatic sections from n=4-5 mice were scored for each experimental group, i.e., Y→Y (non-diabetic donor cells) or D→Y (diabetic donor cells) into non-diabetic (Y) recipients with no mAb treatment, treatment with rat IgG2b or treatment with R1-2.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a treatment including the prevention of insulin dependent (type I) diabetes. More particularly, the invention relates to the use of antibodies to VLA4 in the treatment of diabetes in a prediabetic individual. The term "prediabetic" is intended to mean an individual at risk for the development of diabetes disease (e.g., genetically predisposed) at any stage in the disease process prior to overt diabetes or diabetes onset. The term "diabetic" is intended to mean an individual with overt hyperglycemia (i.e., fasting blood glucose levels ≥ 250 mg/dL). The term "overt diabetes" or "diabetes onset" is intended to mean a disease state in which the pancreatic islet cells are destroyed and which is manifested clinically by overt hyperglycemia (i.e., fasting blood glucose levels ≥ 250 mg/dL).

In the first aspect, the invention provides a method of treatment of diabetes comprising the step of administering a composition capable of binding to, including blocking or coating, the VLA4 antigens on the surface of VLA4-positive cells, including lymphocytes and macrophages. For purposes of the invention, the term "binding to VLA4 antigens" is intended to mean reacting with VLA4 antigens on cells and thereby interfering with interactions between VLA4 antigens and either VCAM-1 or fibronectin on the surface of other cells or thereby inducing a change in the function of the VLA4-positive cells. As demonstrated herein, such binding, including blocking or coating, of VLA4 antigens results in a prevention in or protection against the incidence of diabetes. This demonstration utilized a monoclonal antibody against VLA4 as a binding agent which effectively

blocked or coated the VLA4 antigens. Those skilled in the art will recognize that, given this demonstration, any agent that can bind to, including those that can block or coat, VLA4 antigens can be successfully used in the method of the invention. Thus, for purposes of the invention, any agent capable of binding to VLA4 antigens on the surface of VLA4-bearing cells and which may effectively block or coat VLA4 antigens, is considered to be an equivalent of the monoclonal antibody used in the examples herein. For example, the invention contemplates as binding equivalents at least peptides, peptide mimetics, carbohydrates and small molecules capable of binding VLA4 antigens on the surface of VLA4-bearing cells.

In a preferred embodiment, the agent that is used in the method of the invention to bind to, including block or coat, cell-surface VLA4 antigens is a monoclonal antibody or antibody derivative. Preferred antibody derivatives for treatment, in particular for human treatment, include humanized recombinant antibodies, chimeric recombinant antibodies, Fab, Fab', $F(ab')_2$ and F(v) antibody fragments, and monomers or dimers of antibody heavy or light chains or intermixtures thereof. Thus, monoclonal antibodies against VLA4 are a preferred binding agent in the method according to the invention.

The technology for producing monoclonal antibodies is well known. Briefly, an immortal cell line (typically myeloma cells) is fused to lymphocytes (typically splenocytes) from a mammal immunized with whole cells expressing a given antigen, e.g., VLA4, and the culture supernatants of the resulting hybridoma cells are screened for antibodies against the antigen. (See, generally, Kohler et al., 1975 [40]).

Immunization may be accomplished using standard procedures. The unit dose and immunization regimen depend on the species of mammal immunized, its immune status, the body weight of the mammal, etc. Typically, the immunized mammals are bled and the serum from each blood sample is assayed for particular antibodies using appropriate screening assays. For example, anti-VLA4 antibodies may be identified by immunoprecipitation of ^{125}I-labeled cell lysates from VLA4-expressing cells. (See, Sanchez-Madrid et al. 1986 [41] and Hemler et al. 1987 [42]). Anti-VLA4 antibodies may also be identified by flow cytometry, e.g., by measuring fluorescent staining of Ramos cells incubated with an antibody believed to recognize VLA4 (see, Elices et al., (1990) [43]). The lymphocytes used in the production of hybridoma cells typically are isolated from immunized mammals whose sera have already tested positive for the presence of anti-VLA4 antibodies using such screening assays.

Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium").

Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using 1500 molecular weight polyethylene glycol ("PEG 1500"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridomas producing a desired antibody are detected by screening the hybridoma

culture supernatants. For example, hybridomas prepared to produce anti-VLA4 antibodies may be screened by testing the hybridoma culture supernatant for secreted antibodies having the ability to bind to a recombinant α_4-subunit-expressing cell line, such as transfected K-562 cells (see, Elices et al. [43]).

To produce anti-VLA4 antibodies, hybridoma cells that tested positive in such screening assays were cultured in a nutrient medium under conditions and for a time sufficient to allow the hybridoma cells to secrete the monoclonal antibodies into the culture medium. Tissue culture techniques and culture media suitable for hybridoma cells are well known. The conditioned hybridoma culture supernatant may be collected and the anti-VLA4 antibodies optionally further purified by well-known methods.

Alternatively, the desired antibody may be produced by injecting the hybridoma cells into the peritoneal cavity of an unimmunized mouse. The hybridoma cells proliferate in the peritoneal cavity, secreting the antibody which accumulates as ascites fluid. The antibody may be harvested by withdrawing the ascites fluid from the peritoneal cavity with a syringe.

Several mouse anti-VLA4 monoclonal antibodies have been previously described (see, e.g., Sanchez-Madrid et al., 1986 [41]; Hemler et al., 1987 [42]; Pulido et al., 1991 [44]). These anti-VLA4 monoclonal antibodies such as HP1/2 and other anti-VLA4 antibodies (e.g., mAb HP2/1, HP2/4, L25, P4C2, P4G9) capable of recognizing the α chain of VLA4 will be useful in the methods of treatment according to the present invention. Anti-VLA4 antibodies that will recognize the VLA-α_4 chain epitopes involved in

binding to VCAM-1 and fibronectin ligands (i.e., antibodies which can bind to VLA4 at a site involved in ligand recognition and block VCAM-1 and fibronectin binding) are preferred. Such antibodies have been defined as B epitope-specific antibodies (B1 or B2) (see, Pulido et al. (1991) [36]) and are preferred anti-VLA4 antibodies according to the present invention. The R1-2 antibody used as described herein is a B epitope type antibody.

Human monoclonal antibodies against VLA4 are another preferred binding agent which may block or coat VLA4 antigens in the method of the invention. These may be prepared using *in vitro*-primed human splenocytes, as described by Boerner et al., 1991 [45]. Alternatively, they may be prepared by repertoire cloning as described by Persson et al., 1991 [46] or by Huang and Stollar, 1991 [47]. Another preferred binding agent which may block or coat VLA4 antigens in the method of the invention is a chimeric recombinant antibody having anti-VLA4 specificity and a human antibody constant region. Yet another preferred binding agent which may block or coat VLA4 antigens in the method of the invention is a humanized recombinant antibody having anti-VLA4 specificity. Humanized antibodies may be prepared, as exemplified in Jones et al., 1986 [48]; Riechmann, 1988, [49]; Queen et al., 1989 [50]; and Orlandi et al., 1989 [51]. Preferred binding agents including chimeric recombinant and humanized recombinant antibodies with B epitope specificity have been prepared and are described in co-pending and co-assigned U.S. Patent Application Serial No. 08/004,798, filed January 12, 1993 [52]. The starting material for the preparation of chimeric (mouse V - human C) and humanized anti-VLA4 antibodies may be a murine monoclonal anti-VLA4 antibody as previously described, a

monoclonal anti-VLA4 antibody commercially available (e.g., HP2/1, Amac International, Inc., Westbrook, Maine), or a monoclonal anti-VLA4 antibody prepared in accordance with the teaching herein. For example, the variable regions of the heavy and light chains of the anti-VLA4 antibody HP1/2 have been cloned, sequenced and expressed in combination with constant regions of human immunoglobulin heavy and light chains. Such a chimeric HP1/2 antibody is similar in specificity and potency to the murine HP1/2 antibody, and may be useful in methods of treatment according to the present invention. The HP1/2 V_H DNA sequence and its translated amino acid sequences are set forth in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. The HP1/2 V_K DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 3 and SEQ ID NO: 4, respectively. Similarly, humanized recombinant anti-VLA4 antibodies may be useful in these methods. A preferred humanized recombinant anti-VLA4 antibody is an AS/SVMDY antibody, for example, the AS/SVMDY antibody produced by the cell line deposited with the ATCC on November 3, 1992 and given accession no. CRL 11175. The AS/SVMDY humanized antibody is at least equipotent with or perhaps more potent than the murine HP1/2 antibody. The AS V_H DNA sequence and its translated amino acid sequences are set forth in SEQ ID NO: 5 and SEQ ID NO: 6, respectively. The SVMDY V_K DNA sequence and its translated amino acid sequence are set forth in SEQ ID NO: 7 and SEQ ID NO: 8, respectively.

Those skilled in the art will recognize that any of the above-identified antibody or antibody derivative binding agents can also act in the method of the invention by binding to the receptor for VLA4, and may block or coat

the cell-surface VLA4 antigen. Thus, antibody and antibody derivative binding agents according to the invention may include embodiments having binding specificity for VCAM-1 or fibronectin, since these molecules appear to either be important in the adhesion cells or the extracellular matrix or interfere with traffic of cells through tissues and blood.

Alternatively, the binding agents used in the method according to the invention may not be antibodies or antibody derivatives, but rather may be soluble forms of the natural binding proteins for VLA4. These binding agents include soluble VCAM-1 or VCAM-1 peptides as well as fibronectin, fibronectin having an alternatively spliced non-type III connecting segment and fibronectin peptides containing the amino acid sequence EILDV or a similar conservatively substituted amino acid sequence. These binding agents will act by competing with the cell-surface binding protein for VLA4.

In this method according to the first aspect of the invention, VLA4 binding agents are preferably administered parenterally. The VLA4 binding agents are preferably administered as a sterile pharmaceutical composition containing a pharmaceutically acceptable carrier, which may be any of the numerous well known carriers, such as water, saline, phosphate buffered saline, dextrose, glycerol, ethanol, and the like, or combinations thereof. Preferably, the VLA4 binding agent, if an antibody or antibody derivative, will be administered at a dose between about 0.1 mg/kg body weight/day and about 10 mg/kg body weight/day and at intervals of every 1-14 days. For non-antibody or antibody derivative binding agents, the dose range should preferably be between molar equivalent amounts to these amounts of antibody. Preferably, an

antibody composition is administered in an amount effective to provide a plasma level of antibody of at least 1 μg/ml. Optimization of dosages can be determined by administration of the binding agents, followed by assessment of the coating of VLA4-positive cells by the agent over time after administered at a given dose *in vivo*. Peripheral blood mononuclear cells contained in a sample of the individual's peripheral blood should be probed for the presence of the agent *in vitro* (or *ex vivo*) using a second reagent to detect the administered agent. For example, this may be a fluorochrome labelled antibody specific for the administered agent which is then measured by standard FACS (fluorescence activated cell sorter) analysis. Alternatively, presence of the administered agent may be detected *in vitro* (or *ex vivo*) by the inability or decreased ability of the individual's cells to bind the same agent which has been itself labelled (e.g., by a fluorochrome). The preferred dosage should produce detectable coating of the vast majority of VLA4-positive cells. Preferably, coating is sustained in the case of a monoclonal antibody or monoclonal antibody derivative for a 1-14 day period.

The method of the present invention comprises administering to a prediabetic individual a composition comprising an anti-VLA4 antibody. The examples below set forth the results observed in a rodent model of disease. These results demonstrate a protective effect of anti-VLA4 antibody in disease onset in the acute transfer model of the disease. The non-obese diabetic (NOD) mouse has become an important model of type I or insulin dependent diabetes mellitus since its introduction by Makino et al., 1980 [7] and has been documented as a particularly relevant model for human diabetes (see, e.g., Castano and Eisenbarth [1],

Miller et al., 1988 [5], Hutchings et al., 1990 [33] and references cited therein). That the diabetic syndromes displayed in the NOD mouse and human are similar has been shown by several lines of evidence. For example, in both the NOD mouse and human [1], there is a strong genetic association of diabetes with loci of the major histocompatibility complex. In addition, for example, in both species, an autoimmune pathogenesis is evidenced by (i) the presence of lymphocytic inflammation in the pancreatic islets (i.e., insulitis) that appears to mediate the selective destruction of β cells, (ii) the presence of anti-islet cell antibodies, and (iii) the modulating effects of cyclosporin A. Further evidence in the NOD mouse for an autoimmune etiology of diabetes disease is (i) the ability to transfer diabetes with spleen cells (including purified splenic T cells) from diabetic donors, (ii) prevention of diabetes by *in vivo* treatment with antibodies specific for T cells, and (iii) failure of a thymic nude mice with NOD genetic background to develop moulitis or diabetes (see, e.g., Miller et al., 1988 [5], Hutchings et al., 1990 [33] and references cited therein).

Although the precise events resulting in diabetes remain unclear, in the NOD mouse a progressive inflammatory response in the pancreas appears to be the initial histological lesion which begins as a periductal /perivascular mononuclear cell infiltrate at 3-4 weeks of age. At about 4-6 weeks of age, insulitis may be observed and beginning at about 12 weeks of age, overt diabetes (i.e., consistent values of 1+ or higher using a Testape (Eli Lilly, Indianapolis, IN) assay for glycosuria or greater than 250 mg/dL if plasma glucose is monitored) occurs. To avoid variations in the immune status of the

animals, the NOD mice are obtained from a specific pathogen-free colony and exhibit stable, high incidence of diabetes of about 80% of females and 20% of males which typically become diabetic by about 20 weeks of age. The preferred source for the NOD mice used in the experiments described herein is Taconic Farms (Germantown, NY). A large body of data, particularly from studies of the BB rat and NOD mouse has indicated that type I diabetes may be a T-cell mediated disease. Evidence to date suggests an important role for both major T cell subpopulations (CD4/L3T4 and CD8/Ly2) in the development of diabetes in man and in the NOD mouse. The data supporting the essential role of T cells in diabetes do not exclude the possibility that T lymphocytes may recruit other cells (e.g., macrophages) as the final effectors for β cell destruction. Macrophages have been implicated in the disease process based on their presence in the infiltrated islet and the ability of chronic silica treatment to prevent disease (see, e.g., Hutchings et al., 1990 [33] and references cited therein).

Using the NOD strain of mice, investigators have developed an acute transfer model of disease which parallels the spontaneous disease model in that transferred cells derived from diabetogenic NOD mice mediate the disease process, which is characterized by immune reactive cells that mediate insulitis and islet β cell-specific destruction. Moreover, in this model, certain monoclonal antibodies against T cells (see, e.g., Miller et al., 1988 [5]) and macrophages (see, e.g., Hutchings et al., 1990 [33] have been shown to abrogate disease onset. Such monoclonal antibodies have been used in the treatment of spontaneous disease and adoptively transferred disease, for example, anti-CD4 antibody has

been shown to abrogate disease in both models (Miller et al, 1988 [5] and Shizuru et al., 1988 [30]). Results of treatment with an agent in the adoptive transfer model or spontaneous disease model are indicative of the ability of the agent to modulate the human disease process.

EXAMPLE 1

Effect of Anti-VLA4 Antibody Treatment on Adoptive Transfer of Diabetes

For the adoptive transfer of diabetes experiments, NOD mice were obtained from Taconic Farms (Germantown, NY) or from the Joslin Diabetes Center (Boston, MA). Spontaneously diabetic (D) females of recent onset (13-20 weeks of age) were used as spleen cell donors and 8 week old nondiabetic (Y) females served as recipients. Spleen cells from 4 week old nondiabetic (Y) female donors which fail to transfer disease were used as a negative control.

Recipient mice were placed on acidified water (1:8400 dilution of concentrated HCl in water) one week prior to sublethal irradiation (775 rad) performed in split doses (300 rad, 300 rad, and 175 rad) on each of three days (day -2, -1, and the day of transfer), in order to minimize any incidence of intestinal infection subsequent to high dose irradiation (Gamma Cell 1000 Cesium ^{137} source, Nordion International, Inc., Ontario, Canada). Spleens were harvested from diabetic donors or from nondiabetic controls, cell suspensions made and red cells lysed with Hemolytic Geys solution. Spleen cells were injected intravenously (2-3 x 10^7 in 0.2 ml PBS) pretreated with either 75 μg R1-2 monoclonal antibody (mAb), 75 μg rat IgG2b, or untreated. For the antibody treatment, cells were simply suspended at 1-1.5 X 10^8 cells/ml with mAb at 375 μg/ml and kept on ice until injection. The timing of injection was within 3 hours after last irradiation. Some recipients received PBS alone. The anti-VLA4 mAb R1-2 and isotype-matched rat IgG2b were purchased from Pharmingen (La Jolla, CA). The R1-2 (rat anti-mouse) anti-VLA4 mAb was originally described by Holzmann et al., 1989 [53].

The R1-2 anti-VLA4 mAb blocks VLA4 binding to its ligands (Hession et al., 1992 [54]) and therefore belongs by definition to the B group (Pulido et al., 1991 [44], i.e., is equivalent to anti-human VLA4 mAbs of the B group (e.g., HP1/2 or HP2/1).

The R1-2 mAb or rat IgG2b was administered at a dose of 75 μg/0.2 ml intraperitoneally every 2-3 days, a dosing regimen which was determined to maintain maximal coating of VLA4-positive cells in the peripheral blood, lymphoid organs and bone marrow as detected by staining of peripheral blood cells and single cell suspensions prepared from these organs with a fluorochrome labelled mAb specific for the R1-2 mAb and FACS analysis to measure fluorochrome positive cells (as described above). Injections were maintained through day 12 or day 24 post transfer. Mice were monitored for diabetes by testing for glycosuria with TesTape (Eli Lilly, Indianapolis, IN) and by plasma glucose levels (Glucometer, 3 Blood Glucose Meter, Miles, Inc., Elkhart, IN) and were considered diabetic after two consecutive urine positive tests [Testape values of [+1] or higher] or plasma glucose levels >250 mg/dL.

An inhibitory effect of the anti-VLA4 mAb on the onset of diabetes was demonstrated when spleen cells isolated from NOD diabetic donors were treated with a saturating quantity of anti-VLA4 mAb R1-2 followed by transfer into nondiabetic irradiated hosts, as described above, and the R1-2 mAb was then administered every other day for 12 days in order to maintain maximal coating of all VLA4-positive cells in the peripheral blood and lymphoid organs for two weeks. Figure 1 shows the frequency of recipients that became diabetic and the day

of disease onset for transfer of $2x10^7$ splenocytes from diabetic NOD donor (D→Y) (i) without treatment (closed circles); (ii) with rat IgG2b treatment (closed triangles), and (iii) with R1-2 anti-VLA4 treatment (closed diamonds) as well as for transfer of splenocytes from non-diabetic NOD donors (Y→Y) (open squares). Injection of PBS alone gave 0% incidence. Under these conditions, only 1 of 8 individual R1-2 mAb treated recipients became diabetic, with onset on day 29 post transfer. By contrast, 6/10 and 5/9 individuals became diabetic after receiving splenocytes from diabetic donors treated with no mAb or with non-specific rat IgG2b, respectively. As shown in Figure 1, diabetes onset occurred as early as day 14 post transfer, though administration of the irrelevant rat IgG2b somewhat delayed onset.

These data demonstrate a protective effect of the R1-2 mAb which was dependent upon its specificity for VLA4. Recipients of splenocytes from nondiabetic mice or of PBS alone failed to become diabetic. Thus, treatment with anti-VLA4 antibody reduced the frequency of diabetes during 30 days post transfer.

Although the results shown in Figure 1 demonstrate that clinical diabetes occurred in only 1 of 8 anti-VLA4 treated animals, it was possible that the anti-VLA4 antibody caused only a minor delay in the onset of disease. Plasma glucose levels were monitored in parallel with urine glucose in order to quantify any increase in blood sugar levels and thereby detect progression to clinical disease. In the anti-VLA4 antibody treated group shown in Figure 1, all mice were still normoglycemic on day 29 with an average plasma glucose value of 100 ± 7

mg/dL, n=7, except for the single individual who scored as clinically diabetic by urine test and plasma glucose >500 mg/dL. Thus, disease progression was not apparent in any of the other anti-VLA4 antibody treated recipients shown in Figure 1 on day 29 post transfer, a full 2 weeks beyond the last anti-VLA4 antibody injection. Analysis of sera from these mice confirmed that the anti-VLA4 mAb dropped to low or undetectable levels by day 18-21 post-transfer.

Additional cell transfers were performed in order to confirm that the anti-VLA4 mAb protected against transfer of diabetes. In these experiments, the anti-VLA4 antibody treatment was extended to day 25 post transfer but administered every 3.5 days thereby maintaining saturating levels of R1-2 mAb or rat IgG2b through day 26 when mice were sacrificed for pancreatic tissue. Under these conditions, an inhibitory effect of the anti-VLA4 mAb on the onset of diabetes was also demonstrated upon spleen cell transfer and R1-2 treatment. Figure 2 shows the frequency of recipients (n=4-5 for each group) that became diabetic and the day of disease onset for transfer of $3x10^7$ splenocytes from diabetic NOD donors (D→Y) (i) without treatment (closed circles), (ii) with IgG2b treatment (closed triangles) and with R1-2 anti-VLA4 treatment (closed diamonds), as well as for transfer of splenocytes from non-diabetic NOD donors (Y→Y; open squares). Injection of PBS alone gave 0% incidence. Figure 2 shows that only 1 out of 5 R1-2 mAb treated mice became diabetic by day 22 post transfer whereas diabetes was transferred in 4/4 recipients without R1-2 mAb and 5/5 treated with rat IgG2b. Disease onset occurred as early as day 13 post transfer. These experiments, individually and collectively demonstrate that anti-VLA4 mAb

reproducibly protects against development of diabetes in an acute transfer model of disease.

Further experiments were performed to determine whether the anti-VLA-4 mAb simply delayed disease onset during the treatment period or if it could achieve a longer-term protective effect. Figure 3 shows the onset of diabetes in mice over time after R1-2 injection (once every 3.5 days through day 25) with only 2/5 mice becoming diabetic on days 35 and 38 post transfer, 10-13 days after the last R1-2 injection. By contrast, diabetes occurred in the untreated and IgG2b treated groups as early as day 11 post transfer, with 100% incidence by days 18-21. Surprisingly, disease incidence in the R1-2 treated group did not further increase even as long as 2 months following the last R1-2 injection. Plasma glucose values monitored in parallel during this time reveal that these three individuals were consistently normoglycemic. After this point (i.e., approximately 3 months post-transfer), even the negative control groups which received PBS alone or non-diabetic cells begin developing spontaneous disease. In summary, the VLA-4-specific mAb reduces the incidence of diabetes transfer. Moreover, its protective effect against disease is sustained in the absence of further mAb treatment.

EXAMPLE 2

Effect of Anti-VLA4 mAb on Pancreatis Insulitis

For histological analysis, mice were sacrificed between 2-4 weeks post-transfer as described in this Example and pancreata harvested in 10% formalin buffered saline for paraffin-embedded sections which were stained with hematoxylin and eosin (H&E) for histology. Degree of insulitis was scored as follows: Grade 0: no insulitis [islet devoid of inflammation]; Grade I: peri-insulitis [inflammatory mononuclear cells located peripheral to the islet]; Grade II: <25% infiltrated [<25% of the islet interior contains lymphocytic inflammatory cells]; Grade III: 25-50% infiltrated [lymphocytic infiltration]; Grade IV: >50% infiltrated. The percent of islets in each Grade was then calculated relative to the total number of islets examined. Histologic sections were examined and scored for the degree of insulitis following the adoptive transfer of NOD splenocytes with and without anti-VLA4 mAb treatment and the results tabulated. Specifically, the frequency of uninfiltrated islets (Grade 0-I infiltrate) and islets with Grade II-IV insulitis (as described above) were quantitated. For each experimental group, pancreatic sections from n= 4-5 mice were scored.

Pancreatic tissue was recovered from recipients treated with the anti-VLA-4 mAb for various time periods in order to address its effect on the establishment of islet-specific cellular infiltrates. Mice were treated with nonspecific rat IgG2b or R1-2 mAb every 3.5 days through day 14 when sacrificed. Similarly, mice were treated through day 25 and sacrificed after diabetes was diagnosed or on day 26 post transfer. Mice continuously treated with the R1-2 mAb for 14 days post transfer maintain a high frequency (76%) of uninfiltrated islets,

with only 24% progressing to grade II-IV insulitis. By contrast those treated with nonspecific rat IgG2b show the reciprocal pattern, with 74% severe insulitis. Likewise, in the mice treated with R1-2 though day 25 (20% diabetic, pancreata isolated from mice reported in Figure 2), a high frequency (58%) of uninfiltrated islets were preserved, similar to that (55% uninfiltrated) in nondiabetic recipients of young NOD splenocytes, as shown in Figure 4. By contrast, both the untreated or IgG2b-treated mice had only 28% uninfiltrated islets, and conversely had increased (72%) insulitis. Thus, the anti-VLA-4 mAb treatment appears to specifically inhibit or alternatively to delay the development of insulitis upon adoptive transfer of diabetogenic spleen cells.

In order to distinguish between these alternatives, the pattern of insulitis after 4 weeks post transfer was determined when mice were treated with rat IgG2b or R1-2 mAb through day 12 and then maintained without further treatment. Mice were sacrificed upon diabetes diagnosis or on day 29 post transfer. Analysis of sera from these mice confirmed that circulating anti-VLA-4 mAb dropped to undetectable levels by days 18-21 post transfer. With this protocol, the degree of insulitis in the R1-2-treated group (69% insulitis, 25% diabetic) was similar to that in untreated recipients (73% insulitis, 60% diabetic) though still lower than that in the rat IgG2b-treated mice (96% insulitis, 75% diabetic), as shown in Figure 5. Significantly, the severity of insulitis was similar between the R1-2 treated, untreated and rat IgG2b treated groups with an average of 57%, 47%, 64% Grade III/IV infiltrates, respectively. Even considering only the nondiabetic R1-2 treated individuals, they still exhibited 59% insulitis with 52% Grade III/IV infiltrates.

Recipients of nondiabetogenic NOD splenocytes had only 7% Grade III/IV infiltrates. Conversely, Figure 5 shows that the frequency of uninfiltrated islets was decreased in the R1-2 treated mice as compared to recipients of saline or nondiabetogenic spleen cells. Thus, the degree of insulitis progressed in these R1-2 treated mice (Figure 5) as compared to mice wherein R1-2 treatment was maintained (Figure 4) and approached that in the untreated and rat IgG2b treated control groups. Taken together, these data indicate that anti-VLA-4 mAb administration can delay the progression of insulitis in an acute transfer model of disease.

In summary, anti-VLA4 antibodies were protective against diabetes disease onset (Example 1) and were effective in delaying the progression of insulitis (Example 2) using a murine model for human diabetes. The foregoing examples are intended as an illustration of the method of the present invention and are not presented as a limitation of the invention as claimed hereinafter. From the foregoing disclosure, numerous modifications and additional embodiments of the invention will be apparent to those experienced in this art. For example, actual dosage used, the type of antibody or antibody fragment used, mode of administration, exact composition, time and manner of administration of the treatment, and many other features all may be varied without departing from the description above. All such modifications and additional embodiments are within the contemplation of this application and within the scope of the appended claims.

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The foregoing documents are incorporated herein by reference in their entirety.